A pilot study on Phthalates in Type 2 Diabetes Mellitus (T2DM) patients of South India

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Abstract

Phthalate esters (PAE), the commonly used low volatile, stable plasticizer pollutants are strongly being associated with human pathogenesis. Understanding the regional exposure and determining the influence of PAEs in T2DM enable prevention of T2DM incidence and better disease management. The present study aimed to determine the circulating concentrations of PAEs in a pilot population of healthy volunteers, T2DM patients of South India using standardized GC MS based methods. The study results indicated that in the study population, the BMI of the T2DM patients was significantly higher ($p<0.01$) than the healthy volunteers and among the phthalate esters, DEHP levels of the T2DM patients were significantly higher ($p<0.05$).

Further assessment of the phthalate esters among the male and female study population indicated that the DBP levels were significantly higher ($p<0.05$) in the male population of healthy volunteers and DEHP levels were significantly higher ($p<0.05$) in the male study population when compared to the females. Detailed and large cohort studies will bring to light the influence of PAEs, in particular DEHP and its association with T2DM in the Indian/South Indian population.

Keywords: Phthalate esters, DEHP, DBP, Serum, T2DM patients.

Introduction

The phthalate esters (PAE), often used as plasticizers and extensively used in consumer products including toys, food packaging, cosmetics and medical devices[13,24,25], have received a great deal of attention in recent years owing to their ubiquitous presence and potentially harmful effects on human health, environment. As bio-accumulative components that leak out of consumer items, PAEs can pollute soil, water bodies and sediments and hence fruits, vegetables and sea food consistently carry significant quantities of PAEs[21,37,40]. Despite the prohibitions regarding the usage of di(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP) and butyl benzyl phthalate (BBP) by the European Union[11], they continue to persist in the environment.

As human exposure occurs by all possible routes such as ingestion, inhalation and cutaneous contact, several well-established research studies implicate PAEs as detrimental to human health[11] and prevention of exposure is a significant challenge. Detailed studies have attributed PAE exposure for hormonal deregulation, reproductive, respiratory and immune dysfunctions[14,18,30]. Furthermore, research evidences also indicate the association of phthalates with increased blood sugar levels, insulin resistance and T2DM. In conjunction, direct correlations between the urinary phthalate metabolite concentration and T2DM have been reported[7,9].

PAE exposure is also strongly associated with BMI in children[26,34] and cardiovascular mortality in T2DM patients[42]. In such a scenario, across the globe, several countries continue to actively determine phthalate exposure levels and investigate their association with disease pathogenesis in the regional population. However, in the Indian context, there exists a paucity of PAE exposure and disease incidence/progression. Hence the present study focuses in examining the circulating concentrations of PAEs in a pilot population of south Indian T2DM patients.

Material and Methods

Study participants: 30 randomly recruited healthy volunteers and 30 T2DM patients of Prabhu Diabetes Center (a unit of Prabhu nursing home) were requested to participate in the study. The study was conducted according to standard guidelines with the IEC approval and informed consent from the participants. The study included participants who were healthy male, female volunteers and T2DM patients, aged between 21-85 years and were residents of Tiruchirappalli. The T2DM patients had a fasting blood sugar level above 120 mg/dL, had been diagnosed with T2DM for at least a year and were on therapy with a glucose lowering agent. T2DM patients who were smokers/ non-smokers, with comorbidities, obesity and insulin resistance were also included in the study population. Healthy volunteers/ T2DM patients with a history for T1DM, T2DM and other endocrine dysfunctions such as Acromegaly, Cushing's syndrome and Pheochromocytoma were excluded from the study.

Anthropometric parameters fasting blood sugar levels, blood pressure, family history for T2DM were collected from the patients. For the determination of phthalate esters, 5ml of peripheral blood sample was obtained from the volunteers, T2DM patients. Serum was separated according to standard protocols and utilized for further analysis.

Chemicals and glasswares: The standard EPA phthalate ester mixes (4S8231) and the internal standard phenanthrene-d10 were purchased from Supelco Analytical (Philadelphia, USA) and Cambridge Isotope Laboratories Inc. (USA) respectively. The solvents ethyl acetate and acetonitrile were obtained from Thermo Fisher Scientific India Pvt. Ltd. Utilization of plasticware for the quantitation was avoided in order to minimize errors due to interference and background. Experimental glassware were cleaned based on earlier protocols[2,23]. In brief, all glassware were cleaned using a 10% soap solution (Laboline, Fischer Scientific India Pvt. Ltd., Mumbai) and tap water. The glassware were further cleaned with 50% hydrochloric acid and ultrapure water (ELGA, USA). The rinsed glassware were air dried, wrapped in aluminium foil (Hindalco, Mumbai) and sterilized over night at 200°C in a hot-air oven (Heco, Chennai, India) and utilized for the experiments.

Pre-treatment of samples and GC MS analysis: Pre-treatment of serum samples for phthalate extraction and determination was carried out by using Strata SPE cartridges (30mg, 1ml, Phenomenex Pvt. Ltd., India), after equilibration with acetonitrile and conditioning with 1ml of ultrapure water. The cartridges were further washed with 1ml of 5% acetonitrile, 2ml of 100% acetonitrile and the run through samples were eluted into clean glass tubes. The eluents were then gently streamed with nitrogen until dry at 45 °C. The residues were further reconstituted using 1ml of acetonitrile and utilized for GC MS analysis. Further, quantification of phthalates in the serum sample was carried out using a GC MS system (QP-2010; Shimadzu Corporation, Japan) with an autosampler (AOC-20i) according to standard protocols[2,23].

Results

Monoester metabolites of phthalates are excreted through the urine and are considered to be biomarkers for determining the exposure to phthalates[2]. Since T2DM is a multifactorial disease with a complex etiology, the influence of environmental factors leading to the incidence, pathogenic progression of T2DM is of a greater concern and necessitates thorough, large-scale studies in the local population. Several *in vivo* studies across the globe have associated phthalate exposure with impaired glucose tolerance, increased fasting glucose, hyperinsulinemia and dysfunctions in insulin synthesis, secretion[6,7,9,] Consequently, studies that examine the connection between urine phthalate metabolites and diabetes have brought to light that prevalence of diabetes is significantly related to the urinary phthalate metabolite concentration[6,7,9,10].

Further, understanding the circulating concentration of phthalate metabolites (serum/blood) has been utilized to monitor risk of exposure, regional concentration of phthalates[10,28,32,36]. Earlier studies that assessed the circulating concentrations of DEHP and its metabolites in blood and serum, demonstrated that exposure to phthalates was high in the patients undergoing haemodialysis, owing to risk of exposure from the hemodialyzer, its associated units[32]. In conjunction, recent metabolomic studies that examined the influence of urinary phthalates indicated that phthalates disrupt metabolic homeostasis in children by means of mechanisms such as oxidative stress, lipid metabolism[26,35].

Adjunctively, recent metabolic investigations have also brought to light that circulating phthalate metabolite levels are significantly elevated in T2DM in elderly subjects[28]. However, such studies that examine the association of circulating concentration of phthalates with T2DM are extremely limited in India. Therefore, the present study proposed to examine the influence of serum phthalate esters (DEP, DMP, DBP, DEHP, DnOP) in a pilot population of 30 healthy volunteers and 30 T2DM patient of South India. After the fasting blood sugar level assessment, the prediabetic patients were excluded from the study and the diabetic patients were included in the T2DM study group.

Table 1 of the present study presents the percentage of participants with measurable PAE concentrations. As indicated, a few phthalates such as BBP, DnoP were not measurable in the serum of more than 90 % of the healthy volunteers and patient population respectively. The study results indicate that BBP, DnoP, DEP were quantifiable in 3.5 %, in 14.04%, in 49.12 % respectively, in the study population. In contrast, about 94% of the study participants presented detectable levels of DBP and 100% of the study participants presented detectable levels of DEHP.

Table 1
Detection of Phthalate Esters in the serum of healthy volunteers, T2DM patients

STUDY PARTICIPANTS	DMP	DBP	DEP	BBP	DEHP	DnoP
Healthy Volunteers (N=25)	16	23	24	0	25	8.00
T2DM Patients (N=32)	16	31	4	2	32	0.00
Total (%)	**56.14**	**94.73**	**49.12**	**3.51**	**100**	**14.04**

In sequence, fig.1a presents the mean age, BMI in the healthy, T2DM patients of the study population. The mean age, BMI of the healthy volunteers pertained to 21.92±5.612 and 22.0119±4.13 and 56.00±13.39, 27.35±5.17 in T2DM diabetic patients respectively. It can be observed that the age ($p<0.001$), body mass index ($p<0.01$) varied significantly between the healthy volunteers and T2DM patient population. Further, fig. 1b, presents the percentage of healthy, T2DM patients with other comorbidities and it can be observed that 3.1% of the T2DM patient population presented with comorbidities such as eye, joint and hypertension.

Since obesity is an important risk factor for T2DM, we assessed BMI in the study population and compared the percentage of underweight, normal, over weight and obese individuals across the groups (Figure 2). It can be observed that while only 8% of the healthy volunteers were overweight, about 40.6% of the T2DM patients were overweight. Trending similarly, while only 4% of the healthy volunteers were obese, about 18.75% of the T2DM patients were obese. Further, while 65% of the healthy volunteers were in the normal weight range, only 37.5% of the T2DM patient population were in the normal weight range.

In order to understand the influence of the phthalate esters in T2DM condition, we compared and analyzed the levels of fasting glucose, DMP, DBP and DEHP levels in the healthy and T2DM patients of the present study population (Figure 3). As indicated, the mean±SEM DMP, DBP levels in the T2DM study group were slightly lower (2.35±7.99, 159.3±1.59) in comparison to the healthy participants (3.05±5.06, 238.4±0.89).

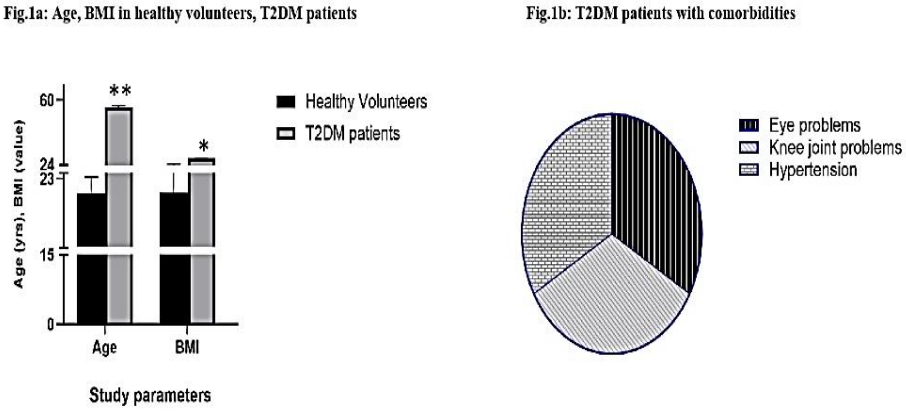

Figure 1: Age, BMI and comorbidities in the study population

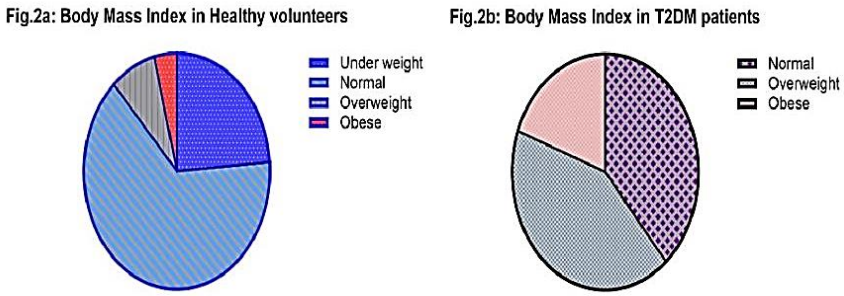

Figure 2: BMI in healthy volunteers and T2DM patients

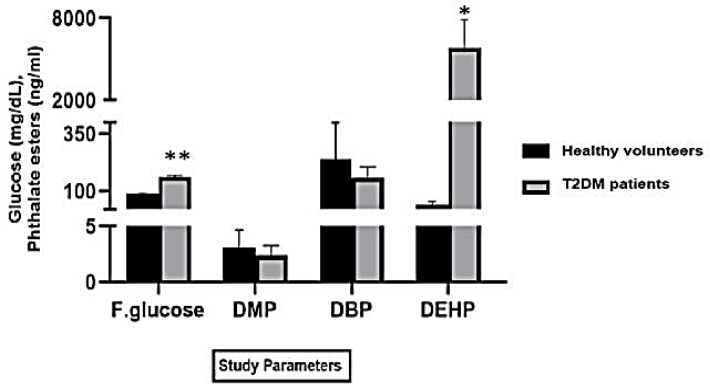

Figure 3: Levels of fasting glucose, Phthalate esters in healthy volunteers, T2DM patients

However, the DEHP levels in the T2DM patients were significantly higher when compared to the volunteers ($p<0.05$). Although a significant association between the assessed phthalates and T2DM (Pearson's correlation analysis) could not be observed in the present study, it can be inferred that assessment with a larger sample size may present with a significantly different study conclusion.

Since, phthalates are also reported to exhibit gender specific differences[18,35], the present study also aimed to assess the gender specific differences in BMI and phthalate concentrations (Figure 4), among the study groups. Figures 4a and 4b present the comparison of the BMI between the male, female population in healthy volunteers, patient population. Fig. 4a presents the mean±SEM of fasting glucose and phthalate concentrations in the healthy volunteer group and it can be observed that the DBP levels of males in the control group were significantly higher ($p<0.05$) than the females.

In terms of the T2DM patient group (Fig. 4b), in conjunction with other earlier studies[9,14,17,46], all the phthalate esters DBP, DMP, DEHP in the present study were higher in the males and DEHP was significantly higher ($p<0.05$) in comparison to the female levels. The study results suggest that the possibility of exposure levels in the males may be higher in the study population.

To further understand the specific influence of DEHP in obesity, we examined the levels of DEHP in underweight, normal weight, over weight and obese individuals. As indicated in figure 5, it can be observed that DEHP levels were higher across all categories (normal, overweight and obese) in the T2DM patients.

Since polluted sources containing high phthalate concentrations are significantly associated with increased risk of exposure, T2DM disease incidence and progression[8,10,17,30,46], the present study further aimed to understand if study participants living near ponds/rivers, working or industrial areas and other possible polluted areas reflected higher circulating phthalate concentrations. Among the study participants 1 healthy volunteer and 7 patients resided near water bodies/industrial areas. The individual data presented in the figure 6, indicates that phthalate ester concentrations in patients are variable and that DEHP values tend to be higher. Despite the fact that pilot study data do not depict actual population representations, they provide a platform to initiate longitudinal studies and to follow up disease incidence, progression, in conditions such as T2DM.

Discussion

Several large-scale studies have led to the understanding that PAE concentrations in humans and livestock vary depending on urban or rural exposure, indoor/ outdoor exposures, occupational exposure from industrial areas and others such as exposure from polluted water bodies.

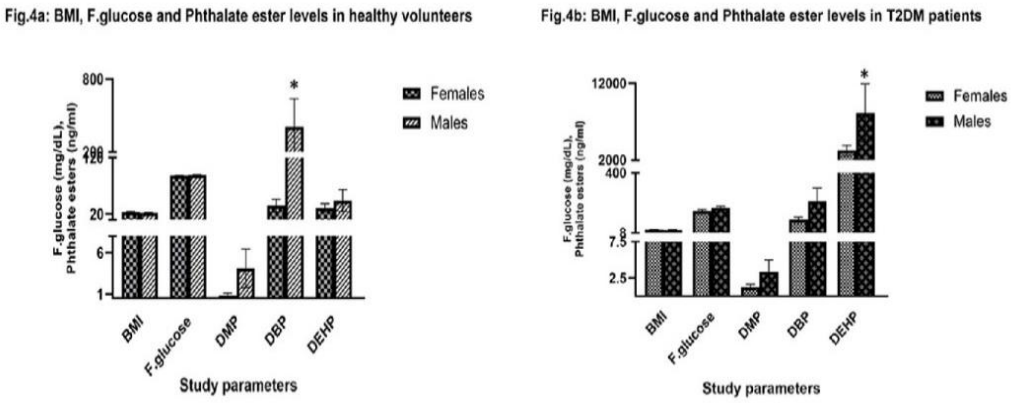

Figure 4: BMI, fasting glucose and Phthalate ester levels in healthy volunteers, T2DM patients

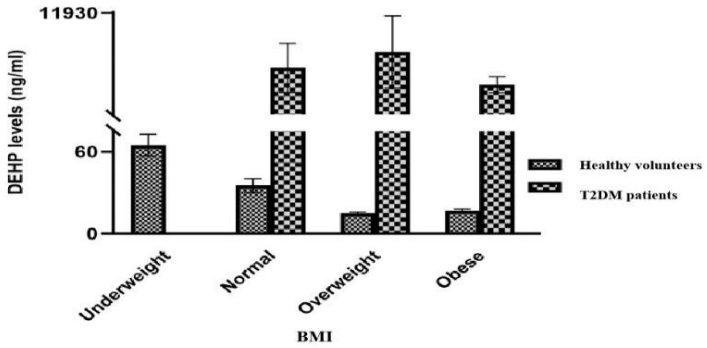

Figure 5: BMI and DEHP in healthy volunteers, T2DM patients

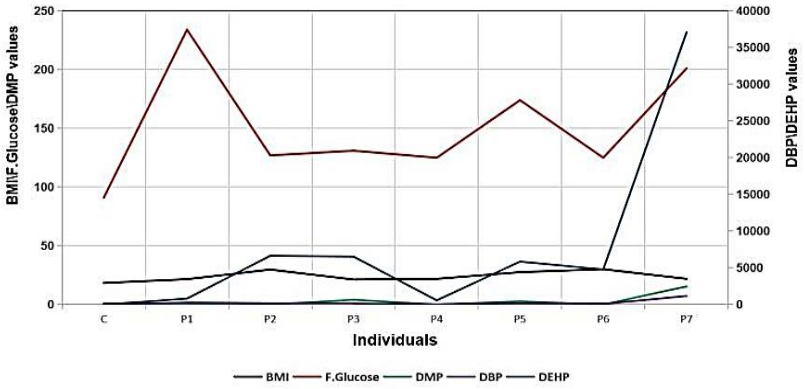

Figure 6: Levels of study parameters in a subgroup of healthy volunteers, T2DM patients residing near rivers/Industrial areas

Further detailed research investigations have also revealed that higher exposure rates pertain to indoor exposure and exposure through food products[11,43] indicating a greater concern to human health[15,40]. Several experimental studies on PAEs and human health have indicated that high molecular weight phthalates such as DEHP significantly associate with early menopause, pre-term and low birth weight[4]. Further, phthalates/DEHP have clearly been documented to influence androgen, estrogen responses, protein secretion, spermatogenesis and effect adverse reproductive outcomes, insulin resistance, T2DM, obesity and asthma[3,5,7,9,10,18,27,28,30,45].

Conjunctively, other studies have also indicated that PAE concentrations correlate with increase in body mass index and pregnancy status[1,3,36,37]. Similarly, other correlative studies have also brought to light that the prevalence of diabetes increased as urinary phthalate metabolites increased and that the risk of cardiovascular mortality in diabetic patients with high phthalate concentrations was significantly increased[1,3,41,43]. Intense research on the effects of PAEs on human health has indicated that exposure to PAEs and the onset of T2DM could be related. While, a vast majority of research studies have focused in determining the urinary phthalate concentrations and their association with T2DM, a few studies have focused in examining the association between the levels of phthalate esters in the blood, serum[25,28,31].

Since there prevails a lacuna in understanding the possible relevance of circulating phthalate concentrations in T2DM in the Indian population, the present pilot study focused in examining the levels of PAEs (DEP, DMP, DBP, DEHP, DnOP) in a pilot population of T2DM patients in South India. Earlier studies have revealed significant levels of PAEs in water, soil/sediment sources in India. Testing for PAEs in the water and sediment samples from the banks of Kaveri River in Tiruchirappalli, revealed that while DEP, DMP were ubiquitous in the sediment samples, DEHP and BBP accounted to 92% of the water samples. Further, among the PAEs examined in these sediments and water samples, it was observed that the concentration of DEHP (1640ng/ml) was the highest[37].

Other studies from Delhi, India have also revealed that PAE exposure increased with air particulate matter in residential areas[32]. Such studies cumulatively indicate that Indians /South Indians, like in other countries are also exposed to significant levels of phthalates that could impact the health and well-being of the residents. Addressing the lacunae that prevails in attempting to understand the association of the PAE concentrations in T2DM in India, the present study focused in understanding the circulating concentration of PAEs in a South Indian pilot population comprising of healthy volunteers and T2DM patients. The results from the present study have indicated that DEHP levels significantly ($p<0.05$) varied in the T2DM study population. Since the volunteers, patients were randomly recruited in the study, age ($p<0.001$) and BMI ($p<0.01$) between the healthy controls and patients differed significantly.

Interestingly in the study population, despite the significant age, BMI variation, only 3.5% of the T2DM study group presented with comorbidities. Although the present pilot study results did not reveal an association of DEHP or other phthalates with T2DM, the significant increase observed in the DEHP levels is strongly suggestive of its detrimental influence in the regional T2DM pathogenesis. Several earlier studies have implicated phthalates in the disruption of blood glucose regulation and insulin resistance[9,10,29,41] and prevalence of diabetes[1,17,41]. Given the fact that both the serum concentrations of phthalates and the urinary metabolite concentrations of phthalates directly relate to exposure levels, it can be suggested that the present study results are in conjunction with earlier studies that have strongly suggested the association of phthalates with T2DM. Other studies examining the association of serum phthalate concentrations in alcoholic T2DM patients have indicated that PAEs such as DiNP, DnBP can also be associated with T2DM [46].

On the other hand, in the light of the significant differences in the age, BMI between the healthy and T2DM patients in the study population, the significant difference in the phthalate concentrations study could also be explained on the basis of variable exposure owing to the age-span, sequestration in the adipose tissues and other important

metabolic factorials. Aging, frailty studies and phthalate concentrations reveal that phthalate metabolite concentrations in older adults are positively associated with frailty[19], indicating that PAE levels could as well increase with age. Contrarily, studies from Germany point out that children[37] may have a greater exposure than adults and may have effective metabolism of the phthalates[26]. Variabilities in phthalate concentrations in terms of gender, racial/ethnic differences have also been reported in several earlier studies that also include the national health and nutrition survey in US that assessed fasting blood glucose, fasting insulin and insulin resistance (HOMA-IR) between 2001-2008[7,9,17,30].

Hand in hand, phthalate metabolite concentrations have strongly been associated with obesity, BMI[3,5,45] and the present study results also demonstrate a substantial increase in the DEHP concentrations across the underweight, normal, overweight and obese categories in the T2DM population. Interesting studies on metabolic/causal relationships of the phthalates have indicated that higher excretion of phthalates could be due to higher retention of phthalates in obese individuals in the adipose tissue[45]. Taken together, it can be speculated that the significant differences in the concentration range of DEHP among the volunteers and the adults could be inclusive of the differences in the exposure, oxidation/metabolism and disrupted glucose metabolism. Since recent studies have iterated that association studies of phthalates in human diseases would be more appropriate when it is based on cumulative exposures[4,24], future longitudinal studies with a large sample size can strongly supplement, document the results of the present study and the role of PAEs in T2DM pathogenesis.

Conclusion

The present study results reveal significantly higher concentrations of DEHP in the T2DM study participants. Further large-scale studies are required to understand the risk of exposure and T2DM incidence, pathogenesis in the regional populations in India.

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